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Kinetic Study on the Inhibition of Hen Brain Neurotoxic Esterase by Mipafox

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JOURNAL ARTICLE

A Kinetic Study on the Inhibition of Hen Brain Neurotoxic Esterase by Mipafox

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Abstract

A direct method of assaying neurotoxic esterase (NTE) activity, using 4-nitrophenyl valerate, has been described. The technique was used to determine the bimolecular rate (k_i), phosphorylation (k_2), and affinity (K_d) constants for the reaction of hen brain microsomal NTE with mipafox. Results indicate that the new technique for assaying NTE makes detailed kinetic studies of NTE inhibition possible. The affinity and phosphorylation constants, K_d and k_2, for the reaction of hen brain microsomal NTE with mipafox, were found to be $6.72 \times 10^{-5} M$ and 3.23 min^{-1}, respectively. The bimolecular rate constant (k_i) for this reaction was obtained by interpreting the results according to two different linear equations. The obtained values for k_i by these interpretations were 4.8×10^4 and $4.0 \times 10^4 M^{-1}$ min^{-1}, respectively.

Introduction

Cresylphosphates and some other organophosphorus esters have induced many cases of neuropathy in humans and other animal species (1-4). Clinical and histopathological evidence suggested that the hen can be accepted as a suitable experimental animal model for screening and studying this kind of neuropathy (1,6-10). Because of the phosphorylating property of the organophosphorus compounds, it is natural to suppose that the neuropathic effect of some of them also may arise from the phosphorylation of an esterase, other than acetylcholinestrase, in the nervous system. Most of the neurotoxic organophosphates were reported as inhibitors of butyrylcholinesterase (BChE) in chickens (11-15). However, the inhibitory effect of these compounds against BChE can not be correlated with their ability to induce neuropathic effect (12,16-18). On the other hand, the nervous system of hens was found to contain many other esterases which can be distinguished by their response to organophosphate inhibitors (18-25). According to their response to paraoxon [0,0-diethyl-0-(4-nitrophenyl)phosphate], a non-neurotoxic agent, and mipafox (N,N′-diisopropyl phosphorodiamidic fluoride), a neurotoxic agent, the phenyl phenylacetate- or phenylvalerate-total hydrolizing esterase activities can be divided into paraoxon-insensitive and paraoxon plus mipafox-insensitive esterases. Differentially, that part of the paraoxon-insensitive esterase activity which is sensitive to mipafox was referred to as neurotoxic esterase, NTE (24-27). Studies *in vivo* and *in vitro* with NTE indicate an acceptable correlation between the neurotoxicity of organophosphorus compounds and their ability to inhibit and age NTE (28-33). Aging means that the inhibited NTE has lost its responsiveness to reactivators (32).

As reported previously, the reaction between an esterase and an organophosphorus inhibitor (*i.e.*, mipafox) appears to progress as shown in Scheme I (34). It was found that the half-life of aging of phosphorylated NTE ranged from less than 1 minute to 10 minutes (32-33). Reactivation of inhibited NTE by potassium fluoride (KF) was possible only immediately after a brief inhibition, and a time-dependent loss of reactivitibility was noted in every case (33). Therefore, the aging phase in Scheme I is highly dependent on Steps 1 and 2 (*i.e.*, formation of the complex and phosphorylation of the enzyme). A kinetic method of assaying NTE using 4-nitrophenylvalerate as a substrate has recently been developed so that a detailed kinetic measurement of NTE inhibition is possible (35). The aim of the present study was to apply this method of assaying NTE in order to determine the affinity, phosphorylation, and bimolecular rate constants for the reaction of mipafox with NTE.

Materials and Methods

Chemicals

4-Nitrophenylvalerate and paraoxon were obtained from Sigma Chemical Company (St. Louis, Missouri) and Aldrich Chemical Company (Milwaukee, Wisconsin), respectively. Mi-

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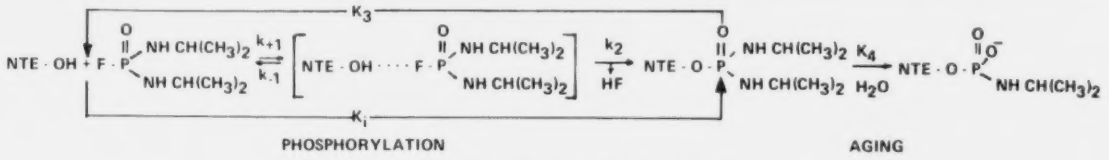

Scheme 1. Phosphorylation and aging of NTE by Mipafox.

pafox was synthesized as reported previously (36,37). Mipafox purity was 99.12% (elemental analysis) and its mp was 58-59°C. Phenylvalerate has a bp of 71-73°C (0.45 mm Hg) and n_D^{18}:1.4868, and its purity was 98.54% (GLC). Elemental analysis results of mipafox and phenylvalerate are presented in Table I. All other chemicals used were of the highest purity grades available from Sigma and/or Aldrich Chemical Companies.

Preparation of the Chicken Brain Microsomal Fraction

Normal adult white leghorn hens (1.5-2.0 kg) were sacrificed by decapitation. Brains (~ 3 g each) were removed as fast as possible and homogenized as 1:10 (w/v) with Tris-HCl buffer (50 m*M*, pH 8.0) containing 0.2 m*M* EDTA for 20 seconds using a Polytron homogenizer with the suitable titanium bits. The microsomal fraction was isolated by differential centrifugation using a Beckman preparative ultracentrifuge (L5-50) with the appropriate rotors. The microsomal pellets were resuspended in the appropriate volume of Tris-HCl buffer containing 0.2 m*M* EDTA and 40 μM-paraoxon in order to obtain a suspension containing 0.5 mg protein per 200 μL. Temperature was kept around 0-4°C during these preparations. The remaining esterase activity following paraoxon treatment was found to be constant for at least 6 hours. The protein content of the resultant microsomal preparation was determined by the method of Lowry *et al.* (38).

Assay of NTE Activity

Paired samples of the above microsomal preparation, each 200 μL, were preincubated in the same paraoxon-treated buffer at 25°C for 20 minutes or the tested time with either (a) buffer or (b) mipafox at 50μM (or the tested concentration) in a final volume of 3 mL. After preincubation, 20 μL of a dimethyl formamidic solution of 4-nitrophenylvalerate or phenylvalerate (25 mg/mL) was added into both tubes and the initial differential rate of hydrolysis was followed in a double beam spectrophotometer, Aminco DW-2, by matching the content of tube (a) against (b) as the reference. Visible 410 nm wave length was used when the substrate was 4-nitrophenylvalerate, while 271 nm of the UV-region was used when substrate was phenylvalerate. Figures 1 and 2 indicate the major practical differences between the orignal Johnson's technique (36) and the new one which was used in this study. There is no need for determination of non-enzymatic hydrolysis using both techniques.

Table I. Elemental Analysis of Mipafox and Phenylvalerate

Compound	Element (%) C	H	F	N	P	Cl
Mipafox						
Calculated for $C_6H_{16}FNOP$	39.56	8.85	10.43	15.37	17.00	0.00
Found (Galbraith)	39.55	8.68	10.20	15.45	16.89	0.06
Phenylvalerate						
Calculated for $C_{11}H_{14}O_2$	74.16	7.87	—	—	—	—
Found (Galbraith)	74.03	7.92	—	—	—	—

Calculation of the Remaining NTE Activity

A model for calculation of the remaining NTE activity is presented in Figure 3.

Evaluation of the Kinetic Constants

Since the reactivation of the phosphorylated NTE is very slow with respect to the aging step, the reaction depicted in Scheme 1 can be represented by the general scheme which was

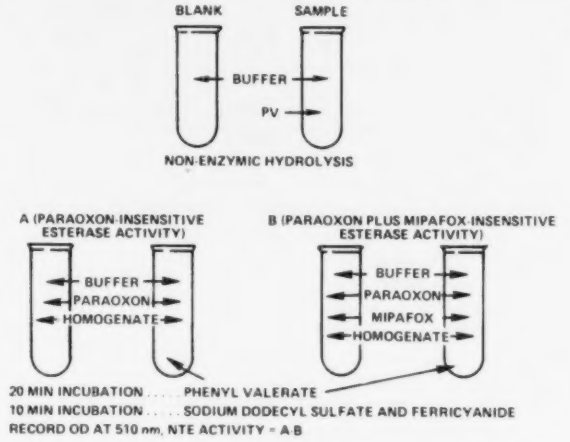

Figure 1. Johnson technique to assay NTE activity.

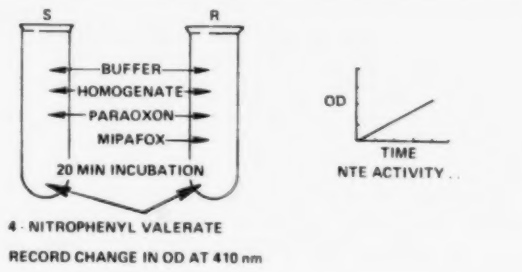

Figure 2. New method to assay NTE activity.

proposed by Aldridge (39) as shown in Equation 1.

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [EI]_a \xrightarrow{k_2} EI \qquad \text{(Eq. 1)}$$

According to this reaction, Wilson (40) and Main (41) have derived an Equation 2, which identifies the values of the constants governing the reversible EI-complex-formation, K_d (*i.e.*, k_{-1}/k_{+1}) and the bimolecular rate constant, k_i.

$$k_i = k_2/K_d = \frac{2.3\,\Delta \log v}{\Delta t}\left(\frac{I}{i} + \frac{1}{K_d}\right) \qquad \text{(Eq. 2)}$$

In this equation, k_2 and K_d are the phosphorylation and the affinity (disassociation) constants, respectively, and v is the measured rate of substrate reaction catalyzed by the esterase remaining uninhibited at time (t) of the inhibition reaction in the presence of the tested inhibitor concentration (i).

Some linear forms of this equation have been used to design experiments and interpret results in order to calculate the values of those constants (41-44). Equation 3 was suggested to overcome some of the difficulties which were experienced (44).

$$\frac{i\,\Delta t}{2.3\,\Delta \log v} = \frac{i}{k_2} + \frac{I}{k_i} \qquad \text{(Eq. 3)}$$

The plot of i against $\frac{i\,\Delta t}{2.3\,\Delta \log v}$ will be linear according to Equation 3. The intercept on the vertical axis is $\frac{I}{k_i}$ while the slope is $\frac{I}{k_2}$ and K_d can be obtained from k_2/k_i.

On the other hand, by following the time-course of inhibition reaction at various inhibitor concentrations, Aldridge (39) previously showed that the reaction was bimolecular and in the presence of excess of inhibitor obeyed an equation of the form:

$$k_i = \frac{2.3\,\Delta \log v}{i\,\Delta t} \qquad \text{(Eq. 4)}$$

It was pointed out that Equation 4 is the limiting case of Equation 2 when $i \ll K_d$ (40).

Interpretation of the results obtained in the present study according to Equation 3 indicates that the measured K_d value was more than any of the tested mipafox concentrations, so that it may also be useful to use the linear form of Aldridge's equation (Eq. 5) to reinterpret the experimental results.

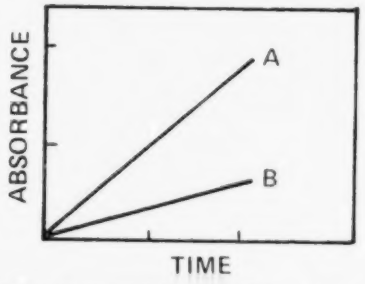

Figure 3. Calculation of the remaining NTE activity. A is total (maximum) mipafox-sensitive (NTE) activity which is determined following preincubation of the paraoxon-treated homogenate with Mipafox at 50 μM for 20 minutes before adding 4-NPV. B is that part of mipafox-sensitive (NTE) activity of an equivalent preparation but preincubated with mipafox at the tested concentration for the tested time. (A-B) is the remaining NTE activity at the condition of the test B.

$$\frac{\Delta t}{2.3\,\Delta \log v} = \frac{I}{k_i\, i} \qquad \text{(Eq. 5)}$$

In this case, the plot of $\frac{I}{i}$ against $\frac{\Delta t}{2.3\,\Delta \log v}$ will be linear and the slope is $\frac{I}{k_i}$.

Results

At the test conditions, the millimolar extinction coefficient for 4-nitrophenol was 11.9; and for phenol it was 1.5, so that the apparent activity of NTE, using phenylvalerate as determined as ΔA/ min/ mg protein, was low compared with that using 4-nitrophenylvalerate. Moreover, working with relatively high concentration of protein affects the sensitivity of the ultraviolet-determinations because of the high absorbance of protein at 270-280 nm. So, using 4-nitrophenylvalerate as substrate was practically more suitable for the direct-like technique. The results were obtained using 4-nitrophenylvalerate as substrate.

Determination of the K_d, k_2, and k_i Values

The first order rate plots obtained with hen brain microsomal NTE pretreated with paraoxon (40μM) and mipafox at different concentrations are presented in Figure 4. The slope of each of the obtained lines was calculated. The slopes obtained are equivalent to the values of the inhibition reaction rates at the corresponding tested mipafox concentrations and from which these rates were obtained and tabulated as in Table II. These slope or rate values are the reciprocal of corresponding $\frac{\Delta t}{2.3\,\Delta \log v}$ values in Equations 3 or 5.

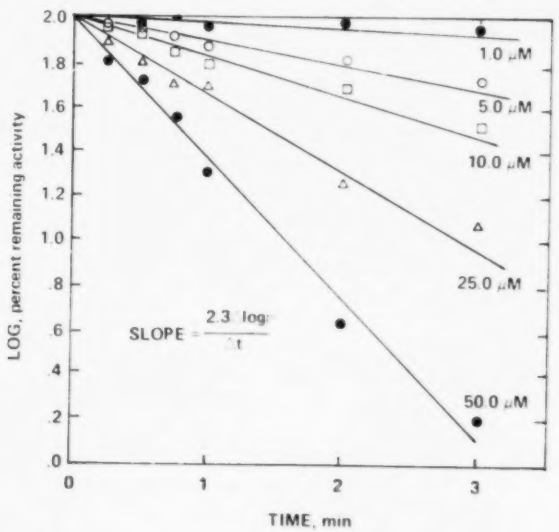

Figure 4. First order rate plots obtained with Hen Brain Microsomal Neurotoxic esterase, pretreated with paraoxon (40 μmM), and Mipafox at different concentrations.

Figure 5 presents a plot for the mipafox concentration (i) against the rate of the inhibition reaction multiplied by the corresponding mipafox concentration ($\frac{\Delta t}{2.3\,\Delta \log \upsilon}$). According to Main-Iverson's equation (Eq. 3), this plot should be linear, and the intercept on the y axis and the slope are $\frac{1}{k_i}$ and $\frac{1}{k_2}$, respectively. The bimolecular rate constant (k_i) for the reaction of mipafox with hen brain microsomal NTE, as obtained from Figure 5, is $4.8 \times 10^4\ M^{-1}\ min^{-1}$. The phosphorylation constant for this reaction as obtained from the slope value is $3.23\ min^{-1}$. From these values, the affinity constant was calculated as $6.72 \times 10^{-4} M$.

The tested concentration of mipafox ranged from 1 to 50 μM. These concentrations are less than the obtained value of K_d. It was of great interest to reinterpret the obtained results according to Aldridge's equation (Eq. 5). Figure 6 presents the double-reciprocal plot of mipafox concentrations against the corresponding inhibition reaction rates. The slope of the linear plot is the reciprocal value of the bimolecular rate constant (k_i). This k_i value was found to be $4.0 \times 10^4 M^{-1}\ min^{-1}$.

Table II. Rates of Inhibition of Hen Brain Microsomal NTE by Different Mipafox Concentrations and the Obtained Rate Constants.

(i) Mipafox Concentration (μM)		Reaction Rate* $\frac{2.3\,\Delta \log \upsilon}{\Delta t}$ (min^{-1})	$\frac{\Delta t}{2.3\,\Delta \log \upsilon}$ (min)	$\frac{i\,\Delta t}{2.3\,\Delta \log \upsilon}$ (M min)
1	1	.046	21.74	21.74
5	0.2	.207	4.83	24.15
10	0.01	.414	2.42	24.42
25	0.04	.874	1.14	28.50
50	0.02	1.380	0.72	36.00

Rate Constants:

$k_i = 4.8 \times 10^4\ M^{-1}\ min^{-1}$ (Eq. 3)

$k_i = 4.0 \times 10^4\ M^{-1}\ min^{-1}$ (Eq. 5)

$k_2 = 6.72 \times 10^{-4} M$

$k_2 = 3.23\ min^{-1}$

*As estimated from the slopes of the lines in figure 1

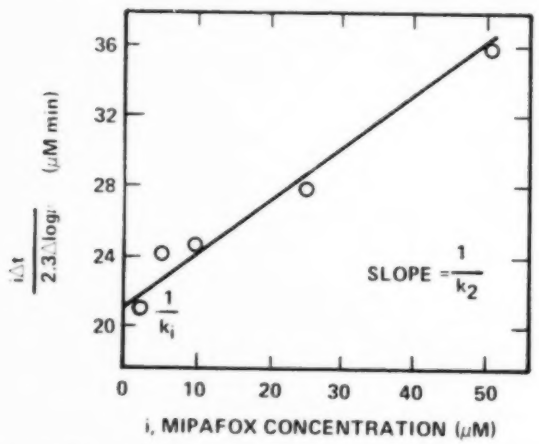

Figure 5. Determination of constant values for the inhibition of hen brain microsomal NTE activity by mipafox, according to Main—Iverson's equation.

Discussion

The obtained results indicate that the reaction of hen brain microsomal NTE with mipafox follows the general reaction of esterases with organophosphates as proposed by Aldridge since 1950. Kinetically, the results fit the equation of Main and Iverson (41) and, for more linearity, they fit that of Aldridge (39) as shown in Figures 5 and 6. These well-fitting interpretations enable us to conclude that mipafox and NTE do form significant reversible complexes as do other organophosphates and esterases. This conclusion is based on the assumption that Equations 3 and 5, with which results are in reasonable agreement, are actually reflecting the proposed mechanism (Eq. 1).

Table II also presents the corresponding values for the bimolecular rate constant and affinity, and phosphorylation constants for the reaction of hen brain microsomal NTE with mipafox as obtained from Figures 5 and 6.

There is no direct evidence in the literature on the value of the bimolecular rate constant (k_i) for the reaction of mipafox with hen brain (or even other source) AChE. However, Lotti and Johnson (45) reported the I_{50} value for the inhibition of hen brain AChE by mipafox as 41.2 μM, which was found to be equivalent to the inhibition reaction for 20 minutes. An approximate predicted value for the k_i of this reaction can be expected from Equation 6 as mentioned by Aldridge and Reiner (34).

$$k_i = \frac{0.695}{t\,I_{50}} \qquad \text{(Eq. 6)}$$

The predictive k_i value for the reaction of mipafox with hen brain AChE evaluated by this equation is $8.43 \times 10^2\ M^{-1}\ min^{-1}$. By comparing the k_i value obtained in the present work for the reaction of mipafox and hen brain NTE (Table II) with the predictive value for the reaction of mipafox and hen brain AChE, the inhibitory power, k_i, of mipafox against hen NTE was found to be approximately 50 times that against hen AChE. Moreover, the low value of K_d reflects high affinity between NTE and the neurotoxic inhibitor, mipafox.

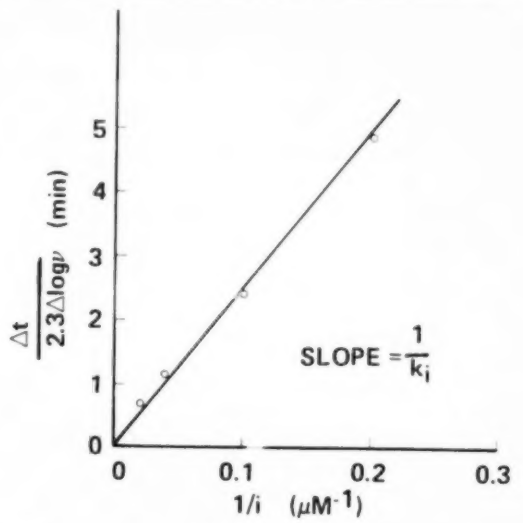

Figure 6. Double-reciprocal plot of mipafox concentrations (i) against the corresponding inhibition reaction rates (Aldridge's equation).

It was proposed that both inhibition of neurotoxic esterase of nervous tissue and subsequent *aging* of the inhibited esterase are necessary events in the pathogenesis of the organophosphate-induced with a number of neurotoxic compounds (32-33). It was reported that the failure to reactivate NTE after inhibition by mipafox is believed to be due to the phosphoramidate structure, which may make the bond from phosphorus to enzyme much more stable, or one C-N-P bond is intrinsically unstable (Scheme 1) in the active-site environment and that aging is virtually instantaneous after inhibition. The present study presents experimental results on the phosphorylation of NTE by mipafox and it also describes a useful technique to study the kinetics of the reaction between NTE and other organophosphorus compounds. It is generally accepted that the inhibitory potency is more properly expressed by the bimolecular rate constant k_i than I_{50} and subsequently pI_{50} values (34, 46). Therefore, the present technique has overcome many of those difficulties which are produced when one starts thinking of using the terminal figure reaction to study the neurotoxicant-NTE interaction kinetically.

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This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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